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Clarke B E; Newton S E; Carroll A R; Francis M J; Appleyard G; Syred A D; Highfield P E; Rowlands D J; Brown F. Improved immunogenicity of a peptide epitope after fusion to hepatitis B core protein. NATURE. (1987 Nov 26-Dec 2) 330 (6146) 381-4.

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Charles I G; Li J L; Roberts M; Beesley K; Romanos M; Pickard D J; Francis M; Campbell D; Dougan G; Brennan M J; et al Identification and characterization of a protective immunodominant B cell epitope of pertactin (P.69) from Bordetella pertussis. EUROPEAN JOURNAL OF IMMUNOLOGY. (1991 May) 21 (5) 1147-53.

Thanks.

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Identification and characterization of a protective immunodominant B cell epitope of pertactin (P.69) from *Bordetella pertussis*

Epitopes defined by monoclonal antibodies (mAb) specific for the *Bordetella pertussis* outer membrane protein P.69 (pertactin) were mapped using a series of amino- and carboxy-terminal deletion mutants expressed in *Escherichia coli*. mAb were found to bind predominantly to a region of pertactin spanning a (Pro-Gln-Pro)$_5$ repeat motif and one mAb was found to bind to another region spanning a (Gly-Gly-X$_{aa}$-X$_{aa}$-Pro)$_5$ repeat motif. To localize further the mAb-binding sites, a panel of synthetic peptides, a series of 94 overlapping hexameric peptides, and a P.69 30-amino acid fusion to a hepatitis B core protein (HBcAg-69), were synthesized. This combined approach has identified the binding site for the mAb BBO5: Pro-Gly-Pro-Gln-Pro-Pro; mAb BBO7, E4A8 and E4D7: Ala-Pro-Gln-Pro-Pro-Ala-Gly-Arg; and mAb BPE3: Thr-Leu-Trp-Tyr-Ala-Glu-Ser-Asn-Ala-Leu-Ser-Lys-Arg. We have used a non-lethal murine respiratory model of *B. pertussis* infection to investigate the ability of a peptide containing the epitope of the mAb BBO5 to elicit protective immunity. Immunization of mice with the HBcAg-69 protein prevented growth of *B. pertussis* in the lungs compared to mice receiving HBcAg alone, and protection correlated with high titers of anti-P.69 antibodies.

1 Introduction

In man, whooping cough is caused by the closely related pathogenic organisms *Bordetella pertussis* and *B. parapertussis*. *B. bronchiseptica* is normally regarded as an animal pathogen, although this organism has also been isolated from humans with a whooping cough-like disease [1]. All three species show phenotypic modulation as a consequence of regulation by the *vir* locus [2, 3]. This locus is known to regulate virulence-related proteins such as pertactin (P.69), filamentous hemagglutinin (FHA), adenylate cyclase, pili and, in *B. pertussis*, pertussis toxin (PTX). We have previously characterized the outer membrane protein P.69 from *B. pertussis* [4]. This antigen can contribute to the protection of mice against an intracerebral [5] or aerosol ([6] and M. Roberts, personal communication) *B. pertussis* challenge. Furthermore mAb against P.69 or against P.68, the immunologically related protein from *B. bronchiseptica* [7], are capable of confering passive protection against a homologous aerosol challenge of *B. pertussis* or *B. bronchiseptica*. These observations suggest that the antigens P.68 and P.69 are important proteins in the protective immune response to the diseases caused by the Bordetellae.

P.69 has recently been shown to bind to mammalian cells, and this binding appears to be mediated, at least in part, by an Arg-Gly-Asp sequence present within the P.69 sequence [4, 8]. Thus, P.69 may be involved in attachment of *B. pertussis* to target cells during the process of infection, or could bind to integrins present on MΦ and leukocytes. On the basis of this observation it has been proposed that P.69 be renamed pertactin [8].

There is considerable interest in the development of acellular pertussis vaccines, and pertactin (P.69), PTX and FHA have all been suggested as possible components of future vaccines [6, 9]. Pertactin has recently been demonstrated to be an agglutinogen [10], and this, in conjunction with its protective properties, stimulates interest in its inclusion in future acellular vaccines.

We have previously reported the cloning and sequencing of the gene encoding the pertactin antigen [4]. Pertactin as extracted from *B. pertussis* appears to be the processed form of a 93-kDa precursor (P.93). This processing appears to occur predominantly near a dibasic Lys-Arg sequence at position 601–602 to produce a protein with a molecular mass of 60.5 kDa that runs anomalously on SDS-PAGE gels at 69 kDa [11].

To map the regions of the P.93 protein that cross-react with mAb, an overlapping series of fusion proteins was expressed in *E. coli* and tested for immunological cross-reactivity. In this report we demonstrate that the repeat protein sequences (Gly-Gly-X$_{aa}$-X$_{aa}$-Pro)$_5$ and (Pro-Gln-Pro)$_5$ of pertactin appear to be immunodominant as identified by the mAb and that the latter sequence is particularly immunogenic, as shown by its ability to confer protection to mice from an aerosol challenge of virulent *B. pertussis*.

[I 9135]

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Abbreviation: **P.69:** Pertactin

2 Materials and methods

2.1 Bacteria and plasmids

E. coli strain TG1 [12], and plasmids pUC8, pUC9, pUC18, pUC19 and M13tg131 have been described [13–15].

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2.2 Antibodies

The mAb BBO5 and BBO7 (IgG_1) raised against the P.68 antigen from *B. bronchiseptica* have been previously described [7], as have mAb BPE3 (IgM), BPD8 (IgG_1) and BPE8 (IgG_1) [10]. mAb D5E9, E4D7, F6E5 and E4A8 (all IgG_1) were generated by injection of BALB/c mice with two doses of pertussis whole-cell vaccine followed by one i.p. injection of a partially purified preparation of FHA from *B. pertussis* 3 days before spleen fusion. Clones were selected by LD and screening against *B. pertussis* whole-cell sonicates. Polyclonal anti-P.69 was prepared by repeated injection of purified pertactin into rabbits with IFA.

2.3 Construction of α-peptide and hepatitis B core antigen (HBcAg) peptide fusion proteins

The 93-kDa open reading frame encoding the P.93 antigen [4] was expressed in *E. coli* as a series of fusions with the α-peptide of *lac*Z in the pUC family of cloning vectors using standard methods [16]. Two plasmids carrying the gene encoding P.93 were used in the initial fusion-protein cloning strategy, pBCS16 and pMLU5. Plasmid pMLU5 contains an oligonucleotide linker with the sites Eco RI-Bam HI-Hind III inserted between the Mlu I sites of pBCS16 as outlined in Fig. 1. Two complementary oligonucleotides: IGCML1 5′-CGCGAAGCTTGGATCCGAATTC-3′ and IGCML2 5′-CGCGGAATTCGGATCCAAGCTT-3′ were kinase labeled, annealed and ligated into Mlu I-digested, gel purified [17] pBCS16 to generate pMLU5 (Fig. 1).

Two overlapping series of constructs were generated (see Sect. 3.1, Fig. 2): series I uses the Sph I site near the signal

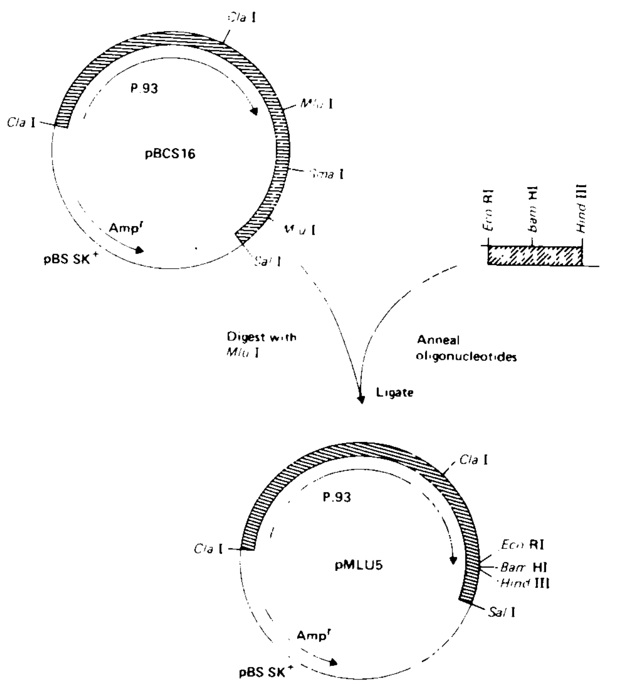


Figure 1. Cloning strategy used to generate plasmid pMLU5. The plasmid pBCS16, containing the DNA sequence encoding the open reading frame for P.93, was digested with Mlu I, the larger fragment gel purified and ligated to the oligonucleotide linker to create pMLU5.

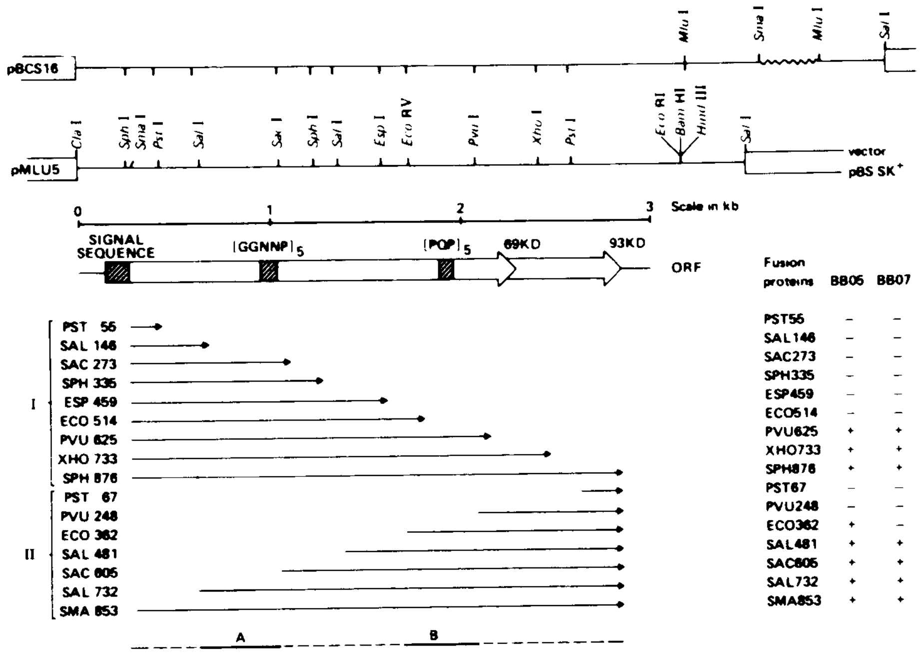
sequence cleavage site of P.93 for the generation of amino-terminal fusion proteins with the α-peptide; all of these constructs were generated in pUC19. The series II constructs maintain the carboxy-terminal region of P.93, and makes use of the family of pUC vectors to generate translational in-frame fusions with the α-peptide. For the series I constructs, fusion plasmid pSPH335 was generated by ligating a 1.0-kb Sph I fragment from pMLU5, containing the 5′-region of the gene for P.93 into pUC19 to generate a *lac*Z-P.93 in-frame protein fusion. By digesting this construct separately with Pst I, Sal I or Sac I and self-ligating the larger gel-purified fragments, the constructs pPST55, pSAC146 and pSAL273 were generated. The full-length *lac*Z-P.93 fusion protein was constructed by double-digesting pSPH335 with Sma I and Eco RI and ligating in the 2.8-kb Sma I-Eco RI fragment containing almost all of the structural gene for P.93 from pMLU5. This full-length *lac*Z-P.93 protein fusion construct was used as a starting point to generate the remaining series I fusion proteins: thus pXHO733, pPVU625, pECO514 and pESP459 were generated by double-digesting the full-length construct pSPH876 with Eco RI and either Xho I, Pvu I, Eco RV or Esp I. The resulting ends were filled in using Klenow and dNTP and the resulting gel-purified fragments ligated to generate pESP459, pECO514, pPVU625 and pXHO733. The series II plasmids pPST69, pSAL481 and pSAL732 were made in pUC8 by ligating Pst I-Hind III or Sal I-Hind III fragments from pMLU5 into Pst I-Hind III- or Sal I-Hind III-digested pUC8. The constructs pECO362 and pSMA852 were in-frame fusions in pUC18 generated by ligating Eco RV-Hind III or Sam I-Sma I gel-purified fragments from pMLU5 and pBCS16, respectively, into Sma I- or Sma I-Hind III-digested pUC18. Plasmid pPVU248 was constructed by ligating a Pvu I-Sma I fragment from pMLU5 into Sma I-digested pUC18. To make pSAC605 as an in-frame fusion, a Sac I-Eco RI fragment from pMLU5 was firstly ligated into Sac I-Eco RI-digested M13tg131. As a consequence a Bgl II-Hind III fragment could be excised that, when ligated into Bam HI-Hind III-digested pUC18, formed an in-frame protein fusion.

The nomenclature of the plasmids refers to the number of amino acid residues of P.93 found in the fusion construct thus, pPST55 is a plasmid that encodes 55-amino acid residues of P.93 fused to the α-peptide of *lac*Z up to a Pst I site, and generates a fusion protein called PST55. *E. coli* cultures carrying these plasmids were induced for expression by growth in L-broth with the addition of isopropyl-β-D-thiogalactopyranoside (IPTG).

An amino-terminal HBcAg fusion protein carrying the pertactin sequence: Met-Ala-Pro-Pro-Ala-Pro-Lys-Pro-Ala-Pro-Gln-Pro-Gly-Pro-Gln-Pro-Pro-Gln-Pro-Pro-Gln-Pro-Gln-Pro-Glu-Ala-Pro-Ala-Pro-Gln-Pro was expressed in yeast using a highly efficient expression system and was purified as described previously [18].

2.4 Synthesis of peptides

Peptides corresponding to the region around the (Pro-Gln-Pro)$_5$ motif in pertactin [4] were synthesized using an adaptation of the method of Merrifield [19] as described by Houghten [20]. The peptides synthesized were: 683 [Ala-



Fusion proteins	Monoclonal antibodies BB05	BB07	BPE3	BPD8	BPE8	D5E9	F6E5	E4A8	E4D7	Rabbit anti-69
PST56	–	–	–	–	–	–	–	–	–	–
SAL146	–	–	–	–	–	–	–	–	–	–
SAC273	–	–	–	–	–	–	+	–	–	+
SPH335	–	–	–	–	–	–	–	–	–	–
ESP459	–	–	–	–	–	–	+	–	–	+
ECO514	–	–	–	–	–	–	+	–	–	+
PVU625	+	+	+	+	+	+	+	+	+	+
XHO733	+	+	+	+	+	+	+	+	+	+
SPH876	+	+	+	–	–	+	+	+	+	+
PST67	–	–	–	–	–	–	–	–	–	–
PVU248	–	–	–	–	–	–	–	–	–	–
ECO362	+	–	–	–	–	–	–	+	–	+
SAL481	+	+	+	+	–	+	–	+	+	+
SAC606	+	+	+	+	+	+	–	+	+	+
SAL732	+	+	+	+	+	+	+	+	+	+
SMA853	+	+	+	+	–	–	+	+	+	+

Figure 2. Line drawing showing the overlapping amino- and carboxy-terminal deletions of P.93. The arrows indicate the extent of expression of each clone. The large arrow represents the P.93 ORF with the putative pertactin cleavage point indicated by an arrowhead. The signal peptide and the two repeat regions are boxed. The regions labeled A and B represent the minimal areas to which mAb binding was detected. The restriction maps for the plasmids pMLU5 and pBCS16 are included showing the restriction sites used to generate the deletion construct. The table on the right shows the reactivity of mAb to the series of fusion proteins.

Pro-Gln-Pro-Gly-Pro-Gln-Pro-Pro-Gln-Pro-Pro-Gln-Pro-Gln-Pro-Glu-Ala-Pro-Ala-Pro-Gln-Pro-Pro-Ala-Gly-Arg-Glu-Leu-Ser-Cys]; 684 [Ala-Gly-Arg-Glu-Leu-Ser-Ala-Ala-Ala-Asn-Ala-Ala-Val-Asn-Thr-Gly-Gly-Val-Gly-Leu-Ala-Ser-Thr-Leu-Trp-Tyr-Ala-Cys]; and 685 [Thr-Leu-Trp-Tyr-Ala-Glu-Ser-Asn-Ala-Leu-Ser-Lys-Arg-Leu-Gly-Glu-Leu-Arg-Leu-Asn-Pro-Asp-Ala-Gly-Gly-Ala-Trp-Gly-Arg-Gly-Cys].

2.5 Solid-phase peptide synthesis and immunoblotting (Pepscan)

Hexameric peptides, overlapping by one amino acid residue, were synthesized on solid-phase polyethylene pins as described [21]. Reactivity of the peptides to mAb was determined by incubation of the pins in antibody for 1–2 h or overnight, followed by washing and incubation in peroxidase-conjugated goat anti-mouse antibody. Enzyme reactivity was determined by incubation of the pins in 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid; ABTS) substrate, and the A_{420} of the solution measured using a Titertek Multiscan MC1100 (Flow Labs., Irvine, Scotland). Pins were sonicated for 30 min at 65 °C in 0.1 M Na_2HPO_4, 0.1% SDS, 0.01 M 2-ME to remove bound antibody and stain complexes prior to incubation in subsequent antibodies.

2.6 Immunization of mice and aerosol challenge

Groups of 5-week-old BALB/c mice were immunized i.m. with 20 µg of HBcAg or HBcAg-P.69 peptide fusion protein (HBcAg-69) with or without IFA. Mice received an identical booster inoculation of antigen 58 days later. Serum samples were taken during the course of the immunization and the antibody response to P.69 and HBcAg were determined by ELISA. Mice were aerosol-infected with *B. pertussis* and the growth of *B. pertussis* within the lungs was determined by performing viable counts of homogenates of lungs removed from infected mice as previously described [22].

3 Results

3.1 Identification of an immunodominant region of pertactin

To map the regions of P.93 recognized by the panel of nine mAb, we constructed a nested set of plasmids expressing an overlapping series of deletions (Fig. 2). For the I-series set of deletions, the amino terminus of each construct was fixed as a translation fusion with *lac*Z at the Sph I site of pUC19. For the II-series set of deletions the carboxy terminus of each construct was fixed and the amino terminus fused with a member of the pUC family of plasmids so as to generate an in-frame translational protein fusion. In a preliminary series of experiments, the recombinant proteins were tested for antibody reactivity by SDS-PAGE followed by Western blotting and/or dot blotting (Fig. 2).

In Western blotting the carboxy-terminal fusion proteins PST67 and PVU248 failed to react with the rabbit polyclonal antibody or with any of the mAb. This is perhaps not surprising as these constructs encode regions found in the P.93 precursor, but not in the mature pertactin antigen [4, 11] and the antibodies were derived from animals

immunized with either *B. pertussis* or purified pertactin. Interestingly, the rabbit polyclonal antibody and a panel of mAb also failed to react with the amino-terminal fusion constructs PST55 and SAL146. These two proteins delineate sequences at the amino terminus of pertactin that appear to be unreactive immunologically.

The data presented in Fig. 2 show that the majority, *i.e.* eight out of nine, of the mAb recognized a region centered around the Pro-Gln-Pro repeat region (labeled B in Fig. 2). This is demonstrated by the reactivity with the amino-terminal fusion proteins PVU625 and the carboxy-terminal fusion proteins ECO362 or SAL481; and by their lack of reactivity with ECO514. Only one of the mAb, F6E5, recognizes a different region as demonstrated by its interaction with the amino-terminal fusion proteins (series I) SAC273, ESP459 and its lack of reactivity with SAL481 and SAC605. Interestingly, this corresponds to a region that encompasses the Gly-Gly-X_{aa}-X_{aa}-Pro repeats (labeled A in Fig. 1), as well as an Arg-Gly-Asp sequence. Such Arg-Gly-Asp sequences have been reported to be involved in cell binding to a variety of adhesion molecules [23, 24]. Recent work [8] suggests that this Arg-Gly-Asp motif in pertactin does have cell adhesive properties and, as a consequence, this region of the molecule may be surface exposed.

The polyclonal rabbit anti-P.69 sera recognized most, but not all of the fusion proteins. Proteins PST67 and PVU248 are not found on the mature pertactin and, therefore, would not be expected to be recognized by anti-P.69 sera. The failure of the proteins PST55 and SAL146 to react could be due to the lack of strong epitopes in the amino-terminal region of pertactin. Alternatively, these proteins could be unstable and be subjected to rapid proteolysis. This is probably the case with SPH355, as this protein does encode an epitope that should be recognized by mAb F6E5.

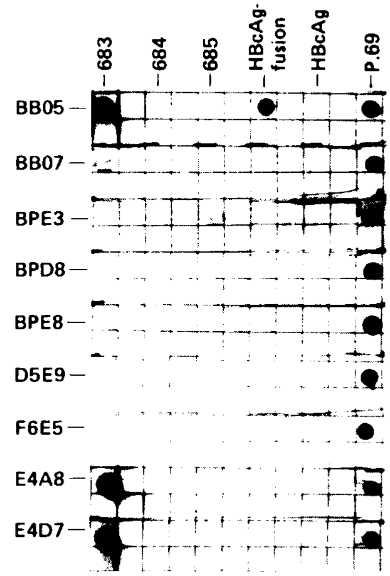


Figure 4. Immunodot-blot reactions. One microgram of each of the test samples 683, 684, 685, HBcAg, HBcAg-69 and pertactin (P.69) was spotted onto strips of nitrocellulose paper. Individual strips were then reacted with the panel of mAb as described in Sect. 2.5.

3.2 Reactivity of mAb with synthetic peptides

To characterize further the protein sequence specific for the binding of certain of these mAb, three overlapping peptides were synthesized corresponding to amino acids 537 to 619 of the pertactin protein (region B in Fig. 1). An additional overlapping sequence was provided by a HBcAg fusion construct expressing the pertactin peptide from residues 537–566 of the mature pertactin protein (Fig. 3). These

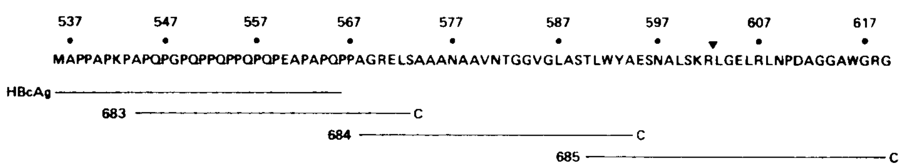


Figure 3. Amino acid sequence between residues 537 and 619 of the mature form of P.93. The underlined sections indicate the extent of the peptides used in this study. The HBcAg-69 protein has an additional Met residue at the amino terminus, while the peptides 683, 684 and 685 each have a carboxy-terminal cysteine residue.

Table 1. Binding of mAb to synthetic peptides, HBcAg fusion protein and purified pertactin[a)]

	mAb	Peptide 683	684	685	HBcAg-69 protein	Purified P.69
B. bronchiseptica	BBO5	+	–	–	+	+
	BBO7	+	–	–	–	+
B. pertussis	BPE3	–	–	+	–	+
	BPD8	–	–	–	–	+
	BPE8	–	–	–	–	+
	D5E9	–	–	–	–	+
	F6E5	–	–	–	–	+
	E4A8	+	–	–	–	+
	E4D7	+	–	–	–	+

a) Binding was determined by dot-blotting as described in Sect. 2.5.

overlapping peptides and the result of immunodot-blots are shown in Fig. 4 and Table 1.

Reactivity of the protective mAb BBO5 occurs in dot blots with HBcAg-69 protein and with the overlapping peptide 683. The mAb BBO7, E4A8 and E4D7 bind only to peptide 683, and not to HBcAg-69 or the overlapping peptide 684. These data suggest that their binding site is likely to occur around the junction point of these peptide sequences (see Fig. 3), *i.e.* the binding site may overlap the sequences at the HBcAg-69/peptide 683 junction. The mAb BPE3 was found to bind to peptide 685, but not to the overlapping peptide 684, suggesting that its binding site lies somewhere within the last 23 amino acid residues of peptide 685 (see Fig. 3), or at the junction of peptides 684/685. Recent data [11] has shown that the carboxy terminus of pertactin is likely to be located near the dibasic Lys-Arg sequences at positions 601–602; therefore, the binding site of mAb BPE3 must be located in the extreme carboxy-terminal region of pertactin.

3.3 Precise mapping of epitopes using synthetic peptides

Six of the mAb were mapped further by Pepscan analysis, *i.e.* a series of overlapping peptides were synthesized on solid-phase supports [21] and their reactivity to a series of mAb tested by ELISA. Ninety-four hexameric peptides were synthesized, covering residues 505–603 of pertactin. Pepscan peptide 1 is Thr^{505}-Asp^{510} and Pepscan peptide 94 is Ala^{598}-Leu^{603}. The reactivity of mAb was determined as described in Sect. 2.5. Three of the mAb, BBO7, E4A8 and E4D7 all bind to only one region around Pepscan peptide 60 (Fig. 5). This is consistent with the data using peptides 683 and 684, and the HBcAg fusion protein (see above). Interestingly, the exact profile of binding of the mAb differ. BBO7 reacts stronlgy with the overlapping Pepscan peptides 59 and 61 (sequences Ala-Pro-Gln-Pro-Pro-Ala and Gln-Pro-Pro-Ala-Gly-Arg), whereas E4A8 and E4D7 react most strongly to only Pepscan peptide 61. The reactivity of BBO7 to Pepscan peptide 43 (Pro-Gly-Pro-Gln-Pro-Pro) could possibly be due to to cross-reactivity with the conserved tetrameric sequence Pro-Gln-Pro-Pro shared between Pepscan peptides 59–61 and Pepscan peptide 43. BBO5 recognizes only Pepscan peptide 43 (sequence Pro-Gly-Pro-Gln-Pro-Pro); this is consistent with recognition of peptide 683 (see above) and locates the BBO5 epitope in the region of the (Pro-Gln-Pro)$_5$ repeats of pertactin. Several peptides were recognized by the mAb BPE8. These peptides were, however, in distinct groups centered around Pepscan peptides 20–34, 41–43, 53–55, 63–65 and 74–76. mAb D5E9 also reacted with a number of peptides but two of these, 19 and 53, which were also reactive with mAb BPE8, were recognized to a greater extent than the others.

3.4 Protective effect of the peptide defined by mAb BBO5

The protective properties of the epitope defined by the mAb BBO5 were investigated in an adult mouse pulmonary infection model using virulent *B. pertussis*. This is a non-lethal model, in which the efficacy of immunogens is assessed by determining the levels of *B. pertussis* in the lungs of infected mice [22].

Table 2. Immunization of mice with HBcAg-69 protein[a)]

Groups	Day 56	Day 65	Day 84	Log CFU in lungs
		Anti-P.69 IgG titer		
HBcAg-69 (IFA)	794	1000	1000	1.85
HBcAg-69	398	501	199	4.68
HBcAg	<10	<10	<10	5.91
		Anti-core IgG titer		
HBcAg-69 (IFA)	12589	31622	39810	–
HBcAg-69	1000	6309	10000	–
HBcAg	1000	10000	31622	–

a) Mice were immunized i.m. with the protein indicated, and the sera analyzed for anti-HBcAg and anti-P.69 antibodies as described in the text. After aerosol challenge with virulent *B. pertussis*, the numbers of bacteria present in the lungs was assayed on day 10.

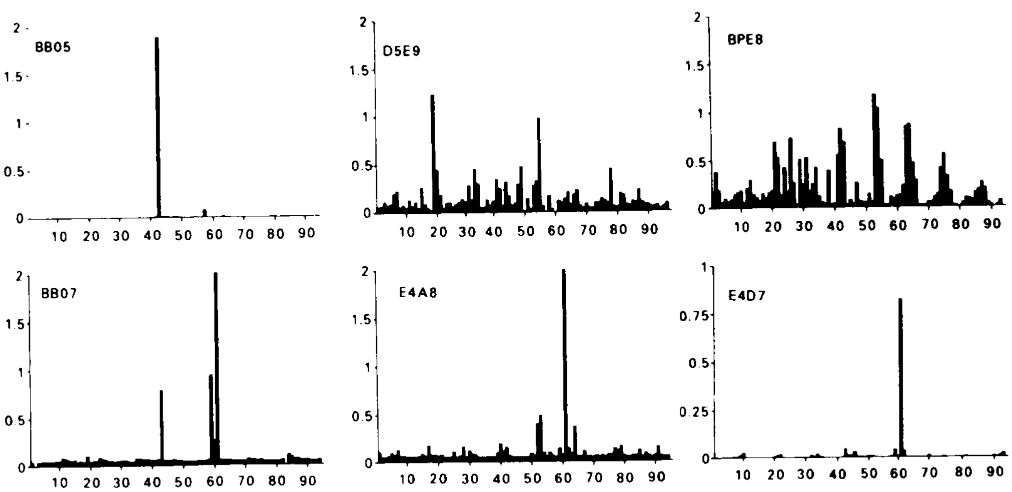


Figure 5. Pepscan analysis of the immunodominant region of pertactin with mAb. The reactivity of each hexameric peptide with the mAb indicated is expressed as A_{420nm} after reaction with second antibody conjugate and stain. The peptide numbering system is described in the text.

Mice were immunized with 20 µg of HBcAg-69 fusion, with or without IFA, or HBcAg (without IFA) on days 0 and 58. All mice were challenged with an aerosol of *B. pertussis* 14 days after the second inoculation. Lungs removed from control mice 1 h after challenge contained 4×10^4 CFU. The numbers of bacteria in the lungs of infected mice were determined 10 days after challenge. The growth of *B. pertussis* in the lungs of non-immunized mice reaches a peak at about this time [22].

Mice immunized with HBcAg-69 without IFA had 10-fold less *B. pertussis* in their lungs than mice which received HBcAg alone (Table 2). A far greater degree of protection was seen in mice given HBcAg-69 with IFA, which had 10000-fold less bacteria in their lungs than HBcAg-immunized mice. Similar levels of reduced colonization are seen in mice receiving *B. pertussis*-derived or recombinant P.69 protein (M. Roberts, personal communication). Mice in all groups had seroconverted against HBcAg prior to aerosol challenge, as judged by their anti-HBcAg IgG titers. Only groups 1 and 2 which received HBcAg-69 developed anti-P.69 titers, the titer in group 1 (with IFA) being 2–5-fold greater than in group 2 (Table 2).

4 Concluding remarks

The *B. pertussis* outer membrane protein pertactin (P.69) has been demonstrated to be a protective antigen [5, 6] and has recently been identified as an agglutinogen [10]. We have mapped those regions of pertactin that are immunologically important by expressing various parts of the molecule in *E. coli* and carrying out Western and dot blot binding experiments using a panel of mAb and a rabbit polyclonal sera. In addition, Pepscan analysis was carried out using a panel of 94 synthetic peptides corresponding to an immunodominant region of pertactin encompassing the $(\text{Pro-Gln-Pro})_5$ repeat sequences. Initial immunoblotting experiments showed that six mAb reactive against the pertactin antigen and two mAb reactive against a related P.68 protein from *B. bronchiseptica* all bound to a region of pertactin flanking and encompassing the $(\text{Pro-Gln-Pro})_5$ repeat sequences (Fig. 2). Some of these mAb have the ability to agglutinate *B. pertussis* cells, suggesting that the proline-rich region of pertactin is surface exposed on the membrane of the bacterial cell.

The remaining mAb reacted with a region of pertactin flanking the $(\text{Arg-Gly-Asp})\text{-}(\text{Gly-Gly-X}_{aa}\text{-X}_{aa}\text{-Pro})_5$ motif. The binding sites of the eight mAb were studied in more detail using three overlapping peptides (683, 684 and 685), an HBcAg fusion protein, and Pepscan analysis. Of the eight mAb apparently reacting with sequences around the $(\text{Pro-Gln-Pro})_5$ region, five were found to bind within regions delineated by the peptides 683, 684 and 685 and the HBcAg fusion. Three of these (BBO7, E4D7 and E4A8) recognized peptide 683, and the Pepscan analysis identified the binding sites of these mAb to the region around the sequence Ala-Pro-Gln-Pro-Pro-Ala-Gly-Arg. The protective mAb, BBO5, was found to bind the HBcAg fusion and peptide 683 and this epitope was localized by Pepscan to the sequence Pro-Gly-Pro-Gln-Pro-Pro. mAb BPE3 was found to bind only to peptide 685, but not to the overlapping peptide 684, and the epitope of this mAb is concluded to lie at the extreme carboxy terminus of pertactin. This mAb was not reactive in the Pepscan assay, perhaps because its binding requires more than six amino acid residues. Of the other mAb found to react around the $(\text{Pro-Gln-Pro})_5$ repeat region, *i.e.* BPD8, BPE8 and D5E9, only BPD8 failed to bind any of the peptides in the Pepscan analysis (data not shown).

The reactivity of mAb BPE8 was fairly complex. While this mAb did not react with the peptides 683, 684, 685 or the HBcAg fusion, the *E. coli* fusion protein analysis suggests that it binds to the $(\text{Pro-Gln-Pro})_5$ repeat region. Pepscan analysis revealed that BPE8 recognizes a number of peptides (Fig. 5). This mAb when administered passively to infant mice, will protect them from a lethal *B. pertussis* challenge [6]. BPE8 has low avidity and it is possible that the protective nature of BPE8 lies in its ability to bind a conformational determinant of pertactin. Thus, the linear sequences identified by the Pepscan analysis could be those involved in the conformational binding of BPE8 to pertactin. This is in contrast to the epitope of BBO5 which is linear (see above). Discontinuous linear peptide sequences which form a conformational epitope recognized by an mAb have been identified previously. Parry et al. [25] characterized the sequences recognized by one neutralizing mAb for foot and mouth disease virus and found the epitope to be composed of two linear but discontinuous sequences.

The protective efficacy of the BBO5 epitope when presented as a fusion to hepatitis core antigen demonstrates the importance of this region of pertactin in immunity. While the greatest inhibition of growth was achieved using adjuvant, a one log drop in colonization was seen using the HBcAg fusion alone. The protection did correlate with antibody titer against P.69, although the response was not linear, possibly indicating that factors other than the IgG response could be involved in the protection conferred by this peptide. Synthetic peptides have been used as vaccines to induce neutralizing antibodies and protective immunity in several animal models of bacterial pathogenicity [26–29]. To our knowledge this is the first example of successful immunization against pertussis infection using a peptide antigen.

The finding that protective mAb specific for pertactin (P.69) are both linear and conformational may have implications in the design of an acellular vaccine against whooping cough. If pertactin is included in any such vaccine, it should be ascertained that the protein used can adopt the correct secondary structure in order that the immune system can induce the production of protective antibodies which recognize both linear and conformational epitopes.

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